

In-situ for 2 genes + nuclear stain in *D. melanogaster*

Reagents

Nuclease Free H₂O
Sigma W4502

PBS packets with Tween 20
Sigma P3563

20X SSC
Sigma S6639

Formamide
Spectrophotometric grade
Sigma 295876

PBT + Tx (1L)
PBS + 0.2% Tween pH7.2 1 bag
20% Triton X-100 10 mL
ddH₂O to 1L

1% w/v BSA in PBT + Tx
BSA : Sigma A7888

Hybe B (200mL)
ddH₂O 200 mL
20X SSC 50 mL
Formamide 100 mL
10% TritonX-100 4 mL

Anti-DIG HRP
Boehringer Mannheim 1207733

Anti-DNP HRP
Perkin Elmer kit NEL747 A001KT

Streptavidin HRP
Perkin Elmer

Coumarin-tyramide
TSA coumarin system NEL703 001KT

Cy3-tyramide reagent pack
Perkin Elmer SAT 704B
enough for 250-750 slides

RNAse A
Sigma R5000
Suspend 100mg lyophilized RNAseA in 10mL
SigmaH₂O + 100µl 1M NaOAc pH5.3. Boil at
100°C 10-30 min. Cool to RT. Add 1mL
TrisHCl pH7.5. Divide into 0.5mL aliquots
~9mg/mL each. Store at -20°C. To generate
working stock, add 500µL 100% glycerol
yielding 4.5 mg/mL in 50% glycerol. Store
working stock at -20°C.

Prepare heat blocks/water baths and probes

Day 1

Prepare an ice bucket and two heat blocks at 55-59°C and 100°C.

Boil probes for ≥3 min and snap cool on ice.

Not all probes require boiling. Those that do not, can be reused 2-3 times. Snap cooling is not essential if you can pipette still boiling probe.

Aliquot embryos

Day 1

Pipet with 1mL blue tips into eppendorf tubes. For 20-50µl settled embryos, adjust with Hybe sol'n to 200µl. For 50-100µl settled embryos, adjust with Hybe sol'n to 300-500µl.

Add probes

Day 1

Warm embryos to 55-59°C.

Add two different probes (either boiling hot or snap cooled):

2 µl/200µl for DIG probes

1-2 µl/200µl for DNP probes

3 µl/200µl for biotin probes

Mix by turning tubes over 2-3 times.

Hybridize overnight (up to 48 hours) at 55-59°C.

Too low a temperature will increase unspecific stain; too high a temperature will damage nuclear morphology. It is better to use the probe for the less reliable/more well-known gene product for the 1st stain.

Preabsorb anti-DIG antibody (if needed)

Day 1

Rehydrate ~50µl of any embryos (good to use those not suitable for staining) by washing 3X with PBT.

Add 1 mL 1% BSA in PBT.

Add 20µl anti-DIG HRP to generate 1:50 stock solution.

Incubate overnight at 4°C while nutating.

Preabsorbed anti-DIG HRP can be stored at 4°C for a couple of months.

Reagents cont'd

Sytox green

Molecular Probes #S7020
1:100 in PBT+Tx, store at -20°C

Coverslips

Fisher 12-548-B 22x22mm #1
Washed in 10% SDS, rinsed in ddH₂O and
100%EtOH and stored in 100% EtOH.

Slides

Gold Seal, Becton Dickinson
Cat No. 3010 3x1" 0.93x1.05mm
Bellco Glass 5638-11013

Mounting Media

DEPEX
Electron Microscopy Services
Cat No. 13514

Wash/block

Day 2

Collect probes for reuse (can be used 2-3 times unless they require boiling), or aspirate to formamide waste.

Wash with Hybe B at 55-59°C

5 min	15 min	15 min	30 min
30 min			

Wash 3X with PBT-Tx at room temp

Wash 4X 20 min with 1% BSA in PBT-Tx at room temp

Add secondary antibodies to RNA for first probe

Day 2

2°Ab for RNA :

for DIG probes : anti-DIG (1:500) 2 hours

for DNP probes : or anti-DNP (1:100) 2 hours

Wash

Day 2

Wash 3X with PBT-Tx (quick)

Wash 6X 20 min with PBT-Tx

The first 3 of these washes shouldn't be longer than 20 minutes or the background will increase.

Wash with PBT-Tx overnight at 4°C.

Overnight wash is essential for anti-DIG HRP, optional for anti-DNP HRP.

1st color reaction to detect first mRNA

Day 3

Aspirate PBT-Tx, leaving 100µl embryos + buffer. If there are more than 100µl embryos, double all volumes.

For every 100µl embryos + PBT-Tx, add 100µl Tyramide amplification diluent.

For every 100µl volume in tube, add 1µl Coumarin-tyramide and mix well.

Nutate tubes at room temp.

Take an aliquot from each tube and place on microscope slide and cover with 22 x 22mm coverslip; one slide can hold 3 samples.

Observe the color reaction under the UV-filter on a fluorescence microscope. When a pattern becomes visible as bright grains, stop the reaction by adding 1mL PBT-Tx.

If there is not a pattern after 1 hour, the staining has probably failed. Stop the reaction at 1hour 15 minutes and continue to determine if it worked too weakly for the eye to detect.

Wash/block

Day 3

Wash 5X with PBT-Tx (quick)

Embryos can be left at 4°C overnight here if there is no time for further steps.

Strip antibodies off embryos

Day 3

Wash with 50% HybeB+Tx in PBT+Tx for 5min at 55°C.

Wash 4X 10-15min with HybeB+Tx at 55°C. Collect the formamide waste.

Wash 3X PBT+Tx, 15min PBT+Tx, Collect the formamide waste.

Nutate embryos for 20min in 5% formaldehyde in PBT+Tx to kill any remaining enzyme. *Time sensitive!*

Wash 3X quickly in PBT+Tx. Collect first wash into formaldehyde waste.

Nutate 30min in PBT+Tx at room temp.

Embryos can be left at 4°C overnight here if there is no time for further steps.

Add secondary antibodies to RNA for second probe

Day 3

If some of the first color reactions failed, take new aliquots to the microscope and inspect them. If there is no pattern even after the excess coumarin-tyramide has been washed away, discard the tubes that failed.

2°Ab for RNA :

for DNP probes : or anti-DNP (1:100) 2 hours

or for biotin probes : streptavidin-HRP 1:100 (1 hour)

Wash

Day 3

Wash 3X with PBT-Tx (quick)

Wash 6X 20 min with PBT-Tx

The first 3 of these washes shouldn't be longer than 20 minutes or the background will increase. If there is no time for further steps, the embryos can be left in the last wash overnight at 4°C.

2nd color reaction to detect second mRNA

Day 3

Aspirate PBT-Tx, leaving 100µl embryos + buffer. If there are more than 100µl embryos, double all volumes.

For every 100µl embryos + PBT-Tx, add 100µl Tyramide amplification diluent.

For every 100µl volume in tube, add 1µl Cy3-tyramide and mix well.

Nutate tubes at room temp.

Take an aliquot from each tube and place on microscope slide and cover with 22 x 22mm coverslip; one slide can hold 3 samples.

Observe the color reaction under the G2A-filter on a fluorescence microscope. When a pattern becomes visible as bright grains, stop the reaction by adding 1mL PBT-Tx.

If there is not a pattern after 1 hour, the staining has probably failed. Stop the reaction at 1hour 15 minutes and continue to determine if it worked too weakly for the eye to detect.

Wash/block

Day 3

Wash 5X with PBT-Tx (quick)

Embryos can be left at 4°C up to 48hours here if there is no time for further steps during the next day or two.

Remove endogenous RNA

Day 3

Bring volume to 500µl with PBT+Tx.

Add 20µl RNase A (4.5mg/ml in 50% Glycerol).

Nutate overnight at 37°C.

Staining nuclei

Day 4

Wash quickly 3X with PBT+Tx.

Nutate for 15min in PBT+Tx at room temp.

Bring volume to 500µl PBT+Tx.

Add 10µl Sytox green (1:100)

Mix sytox well by pipetting vigorously (don't shake), otherwise embryos will not stain properly.

Wrap tubes in foil and nutate at 4°C overnight or up to 48 hours.

Use separate tips for each tube. Because Sytox green is an intercalating dye, it should be treated as a potential carcinogen.

Dehydration/mounting

Day 4

30% : 50% : 75% : 87.5% EtOH in H₂O, 10 min each

3X quick 100% EtOH

Aspirate EtOH, add 75µl xylene/slide to be mounted.

Wipe slides clean with EtOH, layout on paper towels.

Make bridges using #1 coverslips from the box.

Pipet embryos up and down to keep them moving, and add to the slide.

Cover embryos with 350µl DePeX using cut blue tips. Use a different tip for each slide.

Pick a clean coverslip from EtOH with forceps, dry it with lens paper and drop onto sample. Beware of bubbles.

Allow slides to dry 2-4 days in dark.

Even after that, the slides should be kept flat for approximately one month, since the DePeX will flow slowly if the slides are sideways. The slides will be dry enough for staging embryos the following week.

